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EFFECTS OF SALT STRESS ON GROWTH, NODULATION AND NITROGEN FIXATION OF FABA BEAN (*VICIA FABA* L. MINOR) CULTIVATED IN ALGERIA

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ABSTRACT

The aim of this study was to evaluate the effects of 50 and 100 mM NaCl concentrations on growth, nodulation, nitrogen fixation and accumulation of proline of four faba bean (Vicia faba L. minor) cultivars growing symbiotically with Rhizobium leguminosarum bv viciae. The response to salt stress depended on the cultivar and the level salt. Thus, 100mM NaCl caused a great reduction in growth parameters such as leaf area and dry weight of shoot and root, but the height of plant was less affected. Number, dry weight of nodules per plant and nitrogen fixation were reduced with increasing salinity for all cultivars. However, proline content was increased in leaves of all cultivars tested. Sidi Aich and Espresso were less affected by salt than the sensitive cultivars Castel and Maya. Our results suggests that the NaCl tolerance of the Sidi Aich and Espresso cultivars is supported by better growth, the less N2 fixation inhibition and a higher osmotic adjustment.

KEYWORDS: Salt Stress, Cultivars, Dry Weight, Nodules, Nitrogen Fixation, Proline Content

INTRODUCTION

Salinity is considered as a significant factor affecting crop production in arid and semi-arid region of the world (Munns, 2002).

In Algeria, more than 20% of irrigated lands are affected by salinity (Rouabhia and Djabri, 2010). This problem was observed in several regions of the country as in Oran, in certain irrigated perimeters where the salinity reaches considerable proportions (Hamdy, 1999).

An excess of salts in the soil causes many adverse effects on plant growth, which are due to a low osmotic potential of soil solution (osmotic stress), specific ions effects (salt stress), and nutritional imbalances, or a combination of these factors (Ashraf, 1994).

Legumes are classified as salt sensitive crop species (Maas and Hoffmann, 1977) and their productivity limitation is associated with, lower growth of the host plant, poor symbiotic development of root- nodule bacteria (Georgiev and Atkins, 1993) and, consequently, a reduction in the nitrogen-fixation capacity (Soussi et al., 1998; El Nady and Bellal, 2005; Kenenil et al., 2010). Unsuccessful symbiosis under salt-stress may be due to failure in the infection process because of the effect of salinity on the establishment of rhizobia (Fahmi et al., 2011).

Salt stress affects nitrogen fixation in legumes by inhibiting the photosynthate supply to the nodule (Garg and Singla, 2004), reducing supply of respiratory substrates to the bacteroides (Delgado et al., 1994) and by alterations in oxygen-diffusion barrier (Serraj et al., 1994).

Survival and growth in saline environment were the result of adaptative processes, such as ion transport and compartmentation, osmotic solute synthesis and accumulation, which lead to osmotic adjustment (Munns and Termaat 1986). The compounds that accumulate most commonly were proline, trehalose or trigonelline, and glycine betaine, these compounds have been reported to play a role in the response to salt stress of different legumes (Trinchant et al., 2004; Lõpez et al., 2008). The principal agronomic advantage of faba bean is its ability to fix nitrogen by symbiosis with *Rhizobium* bacteria, and thereby substantially contribute to the supply of protein for human food and animal feed and greatly reduce dependence on energy-consuming mineral N fertilizers (Köpke and Nemecek, 2010).

The inclusion in Algeria, of faba bean in the cropping systems is still rather low despite their beneficial function. In order to re-launch this culture, it was essential to identify salt tolerant cultivars to improve agricultural production in soils subject to salinity.

The objectives of this study were to compare the responses of cultivars the faba bean (*Vicia faba* L. minor) to salt stress and to assess their symbiotic performance; this was done by evaluating the plant growth, nodulation, nitrogen fixation, and accumulation of proline.

MATERIALS AND METHODS

Biological Materials and Growth Conditions

The cultivars, Espresso, Maya and Castel, were obtained from GAE SEEDS (France). The local cultivar of Sidi Aich was obtained from Institute of Crop Development in El Khroub, Algeria.

The experiment was conducted in a greenhouse at Technical Institute of Vegetable Crops of Staoueli, Algiers, (3°15’ E, 36°45’ N) under semi-controlled conditions: 28 ± 5°C / 10 ± 2°C day/night temperature, day length ranging from 10-12 h, relative humidity 70 ± 10%.

Seeds were allowed to germinate on filter paper moistened with distilled water for 3 days in darkness. After emergence, seedlings were transplanted into pots filled with 11 kg of cultivated soil with the following characteristics: clay 2.03%; silt 17.51%; sand 78.45%, organic matter 0.68%; total nitrogen 0.03%; phosphorus (ppm) 2.79; pH 7.56.

Five pots were used for each cultivars and saline treatment, with one plant per pot. Three concentrations of salt (0 (no added salt), 50 and 100 mM) in the form of NaCl were added to irrigation water when the first trifoliate leaf appeared (21 days after planting). The treatments were maintained for 8 weeks, coinciding with the onset of flowering.

Harvest

At the end of treatment, the plants were sampled to determine height of plant. Leaf area was measured using an Area meter (Thomson model Air Pax Methatronics).

The harvested plants were separated into shoots, roots and nodules. The dry weight of all plant tissues was estimated after oven drying at 70 °C for 48 h.

Proline Determination

The proline content in leaves was determined using the method of Bates et al. (1973). The concentration of proline was calculated from a standard curve of L-proline.

Nitrogen-Fixation Assay

Nitrogenase (EC 1.7.9.9.2) was determined by the acetylene-reduction-activity (ARA) on the entire root systems of plant, following the method of Hardy et al. (1968). Gas samples were analysed for ethylene produced in the reaction using a Perkin Elmer 8600 gas chromatograph equipped with a flame ionization detector and a 3,2 mm x 2 m (long), Porapak-R-type column (80 mesh) according to Ligero et al. (1986).

Statistical Analysis

The experiment layout was split plot. The parameters studied values were means of five replicates.

Data were analysed using analysis of variance method (ANOVA) and means were separated by least significant difference (LSD) using SPSS statistical software (version 10). The correlation's coefficients among the parameters studied were also calculated.

RESULTS

Growth Parameters

For the four faba bean cultivars, NaCl caused a high significant ($p < 0, 0001$) reduction for all growth parameters (Table 1). However the responses varied with cultivars and with the salt dosage applied.

The percentage reduction relative to the control in height of plant was greater at 100 mM NaCl for Maya and Espresso where it reached around 49 %, followed by Castel and Sidi Aich with 34 % and 36 %, respectively. The leaf area was more sensitive to salinity than height of plant. Cultivars Maya and Espresso showed the largest decrease (74%) in leaf area than other cultivars, especially in the presence of 100 mM NaCl.

Under non-saline conditions, the four cultivars have showed a wide range of dry biomass production. The cultivars Maya and Castel reached the highest shoot (SDW) and root dry weights (RDW), whereas Sidi Aich and Espresso reached the lowest.

The salt treatment with 50 mM NaCl induced a reduction in SDW and RDW about 34% and 43%, respectively, in Sidi Aich cultivar, however in Maya SDW decreased 75% and RDW by 61%. This decrease was even more pronounced with 100 mM NaCl, the smallest reduction was observed in Espresso and Sidi Aich (about 58%) and the greatest was in Castel and Maya (about 84%).

Results of root/shoot dry weight ratio (RSR) are reported in Table 1. For Sidi Aich cultivar, the ratio was constant whereas in Espresso, Maya and Castel, the ratio increased with NaCl,. However, no significant differences were observed between 50 and 100 mM treatments.

Nodulation and Nitrogen Fixation

Symbioses established between *Rhizobium leguminosarum* bv. *viciae* and *Vicia faba* showed significant variation between the four cultivars.

The number of nodules per plant in the absence of salt stress varied between different cultivars (Figure 1a), from 107 nodules in Sidi Aich to 257 nodules per plant in Maya**.** Salt treatment caused a high significant ($p < 0.0001$) reduction of the total number of nodules per plant.

At 50mM NaCl, reduction in nodule's number ranged from 36-52 % for the cultivars Sidi Aich, Espresso and Maya, whilst Castel cultivar had 63 %. In Maya, this parameter was most affected by salinity compared with others cultivars, especially at 100 mM NaCl, in which nodulation was reduced by 75%.

In non- stressed conditions, values of nodule dry weight per plant between cultivars varied in a range of 0.87-1.45 g $plant^{-1}$ (Figure 1b). The cultivar Sidi Aich reached the highest dry weight; this was compensated by producing larger nodules; whereas Maya reached the lowest.

The salt treatment with 50 mM NaCl declined nodule dry weight about 50% in Maya and Sidi Aich, whereas in Castel and Espresso the reduction of this parameter was more than 65%. In addition, in these cultivars, nodule dry weight inhibition was 83-89% with the 100 mM NaCl.

Regarding acetylene reduction activity (ARA), nodules formed by Sidi Aich registered the highest value, and nodules of Castel the lowest value, 39.50 and 24.50 µmol C_2H_4 h^{-1} $plant^{-1}$, respectively (Figure 1c). In general, the lowest salt concentration had a negligible effect on ARA. Sidi Aich appeared to be the less affected cultivar and has showed a reduction of ARA only about 10%; in Espresso and Maya ARA declined about 30%; whereas in Castel the reduction attained 49%. However, highest salt treatment drastically affected ARA, reduction was much higher (84-94%) and all cultivars were salt susceptible.

Proline Determination

In non- stressed conditions, all the cultivars showed similar proline content in leaves (on average 0.22 mg .g leaf fresh $weight^{-1}$) but under salt stress conditions a high significant ($p < 0.0001$) increase of proline content was observed (Figure 2). However, no significant differences were observed between 0 and 50 mM treatments in Castel, Maya and Sidi-Aich.

With 100 mM NaCl, the highest proline content were observed in Sidi Aich and Espresso cultivars (1.28 and 1.13 mg .g leaf fresh $weight^{-1}$ respectively) whilst Castel and Maya accumulated 0.74 and 0.61 mg .g leaf fresh $weight^{-1}$ respectively.

DISCUSSIONS

In the present study, we have evaluated the responses to salt within four cultivars of faba bean in symbiosis with *Rhizobium leguminosarum* bv *viciae.* The responses were dependent on the cultivar and the level of applied salt.

Our results, showed a reduction of all parameters of growth of four cultivars under saline conditions (Table 1). Indeed, with 100 mM of NaCl, (after 8 weeks), plant dry weight has decreased by about 56-58 % in Sidi Aich and Espresso respectively, and by about 84 % in Maya and Castel. Similar results were reported in other faba bean cultivars. Cordovilla et al. (1999) found that plant dry weight in *Vicia faba* L. cv. Alborea has showed reductions of 65% at 100 mM NaCl (after 6 weeks). Also, the biomass of shoots in *Vicia faba* L. cv. Scirocco was reduced to 67% compared to control plants after 9 day application of 100 mM NaCl (Pitann et al. 2011).

According to Aslam et al. (1993) both fresh and dry weight could be used as the criterion for assessing relative salt tolerance. The comparison of these percent inhibitions, suggested that Sidi Aich and Espresso cultivars are less sensitive to saline conditions than Maya and Castel.

Ullah et al. (1993) reported that, the reduction in dry matter production of faba bean may be caused by ion toxicities, reduction water absorption, impaired biochemical and physiological processes associated with increasing salt stress.

For all cultivars, salinity affected leaf area more than plant height (Table 1). Tammam (2003) proposed that the death of old leaves dues to ion-toxicity might prevent the supply of nutrients and hormones to young leaves. Under saline conditions, the increase in root-shoot ratio (RSR), (Table 1) were even more significant in Castel, Espresso and Maya cultivars, that indicated that shoot growth was more inhibited by NaCl than root growth. In this regard, Tejera et al. (2006) has suggested that increases of RSR with salinity could be associated with lower shoot growth and therefore root growth might occur at the expense of the shoot growth.

In all the cultivars, the number of nodules and their dry weight were reduced by salt concentrations. These results are in accordance with experiments of Cordovilla et al. (1999) in faba bean, with Saadallah et al. (2001) in common bean and with Ben Salah et al. (2011) in *Medicago ciliaris*. Our results showed that, the greatest reduction was observed in Castel and Maya (Figure 1a, b).The reduction of nodule dry weight was significantly larger than the number of nodule. Similar results were reported by Bouhmouch et al. (2005) where the decrease in nodule number caused by 25mM NaCl on two common bean cultivars, was about 50% and about 94% in nodule weight. The reduction of nodule's number suggested, that the salt inhibited the initiation and the development of a second generation of nodules, since the salt stress was applied after nodulation (Saadallah et al. 2001; Ben Salah et al. 2011).

Salinity also decreased, symbiotic nitrogen fixation; and the most affected salt concentration was 100mM NaCl (Figure 1c). Similar findings have been reported in *Vicia faba* (Delgado et al.1994; Cordovilla et al. 1999), *Cicer arietinum* (Garg and Singla, 2004; Tejera et al. 2006) and *Phaseolus vulgaris* (Khadri et al., 2007). The nodules of Sidi Aich and Espresso were more effective in comparison to other cultivars.

The decrease in nitrogen fixation may be due to the inhibition of the carbon flow from the plant to the bacteroids (Garg and Singla 2004; Lõpez et al. 2008) and a reduction in cytosolic protein production, specifically leghemoglobin by nodules (Delgado et al. 1994). Serraj et al. (1994) attributed the decrease in nitrogen fixation by salt stress, to limitation of oxygen diffusion in nodules or to the toxic effects of Na or Cl accumulation. The correlations among dry weight of the whole plant, dry weight of nodules and nitrogen fixation per plant were positive and significant in all cultivars (Table 2). These correlations indicated a close relationship between plant growth and symbiotic nitrogen fixation of nodules.

The proline levels of salt stressed plants were significantly higher with 100 mM NaCl compared to the control (Figure 2). Sidi Aich and Espresso were less sensitive to salt accumulated much more proline (5-6 folds over non saline conditions) than Maya and Castel most sensitive (only 2 of 3 fold higher proline than control plants). The increased accumulation of proline was reported in many species exposed to salinity (Dimartino et al. 2003),and this accumulation has been proposed to be part of the process of osmotic adjustment that contributes to the cellular adaptation of many plant species to drought, salinity, and other stresses (Munns and Termaat, 1986). In cowpea (*Vigna unguiculata*), a salt tolerant cultivar 'Pitiuba' has a higher osmotic adjustment as compared with a salt sensitive cultivar 'Perola' (Freitas et al. 2001).

In contrast, Katerji et al. (2002) could not find relationship of osmotic adjustment with salt tolerance of broad bean (*Vicia faba* L.). Thus, controversies exist to whether accumulation of proline was essential for improving salinity tolerance or, whether it was just a symptom of salt tolerance.

CONCLUSIONS

This study showed variability among faba bean cultivars in the response to salt stress. The responses were dependent on the cultivar and the level of applied salt.

The cultivars Maya and Castel were most salt-sensitive cultivars whereas Sidi Aich and Espresso were relatively tolerant. These cultivars showed at high salt concentration better growth, less N_2 fixation inhibition and a higher osmotic adjustment; as such they could be the choice for field applications.

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APPENDICES

Table 1 Effect of NaCl treatments on plant height (cm) , leaf area (cm^2) ,shoot dry weight (SDW), root dry weight (RDW), plant dry weight (PDW) in g $plant^{-1}$ and root-shoot ratio (RSR) of four faba bean cultivars treated with 0, 50 and 100 mM NaCl.

Table 2 R values showing correlations between dry weight of the whole plant (PDW), nodule dry weight (NDW) and acetylene reduction activity (ARA) in faba bean cultivars treated with 0, 50 and 100 mM NaCl.

Figure 1 Effect of NaCl treatments on nodule number (a), nodule dry weight (b) and on acetylene reduction activity (ARA) (c) of four faba bean cultivars treated with 0, 50 and 100 mM NaCl. Mean values of 5 replicates are followed by ± S.E.

Figure 2 Effect of NaCl treatments on the proline content (mg .g leaf fresh $weight^{-1}$) of four faba bean cultivars treated with 0, 50 and 100 mM NaCl. Mean values of 5 replicates are followed by ± S.E.

Table 1

Cultivars	Nacl (Mm)	Plant Height	Leaf Area	SDW	RDW	PDW	RSR
Castel	0	44.6 ±2.08[a]	35.8±3.37[a]	26.36±1.56[a]	6.22±0.56[a]	32.58±1.40[a]	0.23±0.03[b]
	50	34.2±3.83[b]	22.8±3.15[b]	8.08±0.7[b]	3.76±0.14[b]	11.85±0.77[b]	0.47±0.04[a]
	100	29.2±2.95[b]	13±1.76[bc]	3.35±0.25[c]	1.63±0.13[c]	4.98±0.33[c]	0.49±0.04[a]
Espresso	0	50.4±3.48[a]	38.8±2.88[a]	14.54±0.43[a]	6.24±0.45[a]	20.78±0.85[a]	0.42±0.01[b]
	50	37.8±1.24[b]	14.2±1.15[b]	7.43±0.56[b]	5.43±0.44[a]	12.87±0.80[b]	0.73±0.07[a]
	100	25.6±1.74[bc]	9.8±1.88[b]	4.57±0.16[c]	4.06±0.49[b]	8.63±0.46[c]	0.89±0.13[a]
Maya	0	57.6±1.63[a]	59±7.19[a]	24.11±2.20[a]	8.33±0.33[a]	32.45±2.28[a]	0.35±0.04[b]
	50	40±2.66[b]	23.6±1.12[b]	5.9±0.26[b]	3.23±0.42[b]	9.13±0.51[b]	0.54±0.07[a]
	100	30.2±1.62[c]	15±2.25[b]	3.17±0.29[c]	2.22±0.16[c]	5.4±0.38[c]	0.71±0.05[a]
Sidi Aich	0	66.2±4.63[a]	27.8±1.11[a]	13.11±0.27[a]	5.01±0.26[a]	18.12±0.34[a]	0.37±0.02[a]
	50	53.2±2.41[b]	17.6±2.13[b]	8.53±0.41[b]	2.85±0.23[b]	11.39±0.46[b]	0.33±0.03[a]
	100	41.8±1.11[c]	9.6±1.28[c]	5.48±0.13[c]	2.38±0.29[b]	7.87±0.33[c]	0.43±0.04[a]

Means with the same letter in the vertical columns do not differ significantly (P≥0.05).Means are followed by ± S.E.

Table 2

R Values Comparisons	Castel	Maya	Espresso	Sidi Aich
PDW & NDW	0.94**	0.88**	0.86**	0.89**
PDW & ARA	0.92**	0.74**	0.83**	0.80**
NDW &ARA	0.91**	0.76**	0.71**	0.79**

*P< 0.05, ** P<0.01.

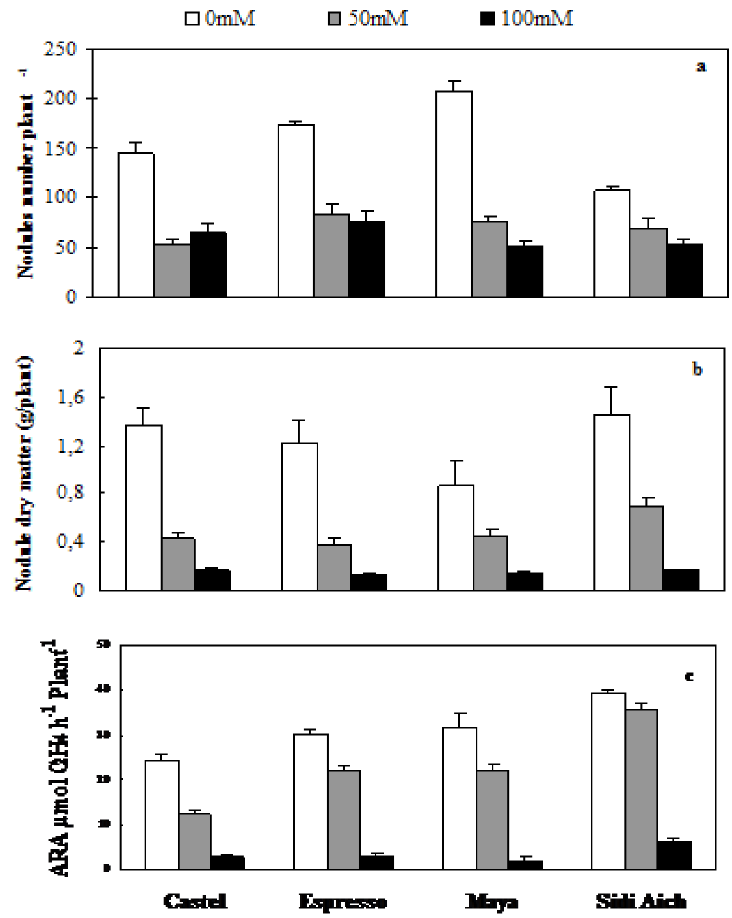

Figure 1

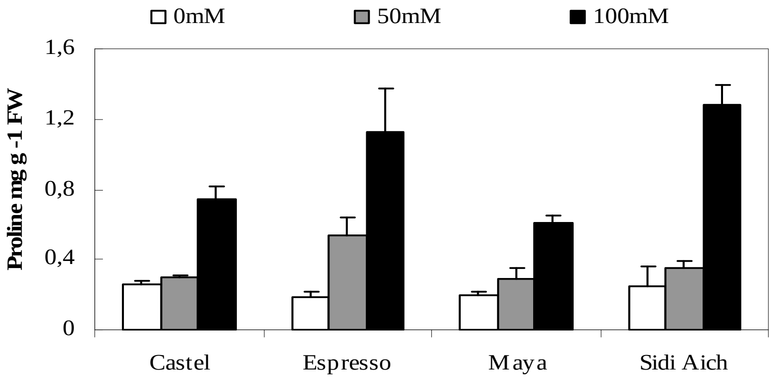

Figure 2